SPECIFICATION

NUCLEIC ACID CONSTRUCT CONTAINING FULLLENGTH GENOME OF HUMAN HEPATITIS C VIRUS, RECOMBINANT FULLLENGTH VIRUS GENOME-REPLICATING CELLS HAVING THE NUCLEIC ACID CONSTRUCT TRANSFERRED THEREINTO AND METHOD OF PRODUCING HEPATITIS C VIRUS PARTICLE

TECHNICAL FIELD

The present invention relates to nucleic acid constructs containing full length genome of hepatitis C virus, an in vitro method for producing hepatitis C virus particles and use of the produced hepatitis C virus particles.

BACKGROUND ART

Hepatitis C virus (HCV) belongs to the family Flaviviridae and is a virus having a single stranded (+) sense RNA genome and is known to cause hepatitis C.

HCV causes chronic hepatitis by persistent infection. Currently, the main cause of chronic hepatitis observed worldwide is persistent HCV infection. Actually, around 50% of individuals with persistent infection develop chronic hepatitis. Chronic hepatitis in approximately 20% of these patients shifts to liver cirrhosis over the course of 10 to 20 years, and some of these patients further go on to advanced lethal pathological conditions such as hepatic cancer.

Hepatitis C is currently treated mainly by a therapy using interferon-α or interferon-β, or a therapy using a combination of interferon-α and ribavirin, a purine-nucleoside derivative. However, even when these therapies are performed, the therapeutic effects are observed in only approximately 60% of all treated patients. When therapies are ceased after effects are seen, the disease recrudesces in more than half of the patients.

It is an important goal to develop therapeutic agents or prophylactic agents effective

against hepatitis C. The incidence rate of hepatitis C, which in the end brings about serious consequences, is high in industrial countries, and there is currently no causal treatment available. Hence, the development of HCV-specific chemotherapies and vaccine therapies are desired. A target for the development of an anti-HCV agent may be the suppression of HCV replication or the suppression of infection of cells with HCV.

Recently, HCV subgenomic RNA replicon systems have been prepared as HCV-derived autonomously replicable RNA (see, Patent Documents 1, 2 and 3, Non-Patent Documents 1-4). In the HCV subgenomic RNA replicon systems, HCV replicon RNA in which the structural genes of the HCV genome is eliminated and replaced with a drug-selectable marker gene, are prepared and introduced into cultured cells, and thereby the replicon RNA is replicated autonomously in the cells. By using these systems it becomes possible to analyze the replication mechanism of HCV. However, this is an experimental system in which only viral RNA replication is evaluated in the process of the proliferation and replication of HCV virus, and the process of the formation of HCV virus particles in the infected cells and the extracellular release or infection to another cell cannot be analyzed.

At this time, the process of HCV virus particle formation and extracellular release as well as infection to another cell can only be evaluated in the experimental systems using animals such as chimpanzees (Non-Patent Document 5). However, the experimental systems using living organisms such as animals are complicated and very difficult to analyze. Therefore, in order to analyze the process of HCV virus particle formation and extracellular release as well as infection to another cell, and to produce an anti-HCV agent which will have the action mechanism of inhibiting this process, it is necessary to establish a highly simplified experimental system reproducing this process, i.e. a HCV virus particle production system in cell culture experimental systems.

Further, once HCV virus particles can be provided stably using the cell culture system, it is possible to attenuate the virus or to produce noninfectious HCV virus using molecular biological techniques, and this can be used in vaccines.

Patent Document 1: JP Patent Publication (Kokai) No. 2001-17187

Patent Document 2: International Patent Application PCT/JP03/15038

Patent Document 3: JP Patent Application No. 2003-329082

Non-Patent Document 1: Lohmann et al., Science, (1999) 285, p. 110-113

Non-Patent Document 2: Blight et al., Science, (2000) 290, p. 1972-1974

Non-Patent Document 3: Friebe et al., J. Virol., (2001) 75(24): p. 12047-12057

Non-Patent Document 4: Ikeda et al., J. Virol., (2002) 76(6): p. 2997-3006

Non-Patent Document 5: Kolykhalov et al., Science, (1997) 277, p. 570-574

Non-Patent Document 6: Kato et al., Gastroenterology , (2003) 125, p.1808-1817

Non-Patent Document 7: Yanagi et al., Proc. Natl. Acad. Sci., (1997) 96(16): p.8738-8743

Non-Patent Document 8: Okamoto et al., J. Gen. Virol., (1991) 73, p 2697-26704

Non-Patent Document 9: Aoyagi et al., J. Clin. Microbiol., (1999) 37(6): p.1802-1808

DISCLOSURE OF THE INVENTION

The objective of the present invention is to provide a method for efficiently replicating RNA containing full length HCV genomic sequences and a method for producing HCV virus particles containing full length HCV replicon RNA or full length HCV genomic RNA in a cell culture system. The objective of the present invention has never been achieved so far.

As a result of intensive studies to achieve the above object, the present inventors have developed a method for producing HCV virus particles in a cell culture system. That is, the present invention is as follows.

[1] A replicon RNA, comprising a nucleotide sequence comprising a 5' untranslated region, a core protein coding sequence, an E1 protein coding sequence, an E2 protein coding sequence, an NS2 protein coding sequence, an NS3 protein coding sequence, an NS4A protein coding sequence, an NS4B protein coding sequence, an NS5A protein coding sequence, an NS5B protein coding sequence, and a 3' untranslated region of genomic RNA of hepatitis C virus of genotype 2a, at least one selectable marker gene and/or at least one reporter gene, and at least one IRES sequence.

In this replicon RNA, preferably the nucleotide sequence comprises the 5' untranslated region, the at least one selectable marker gene and/or the at least one reporter gene, and the at least one IRES sequence, and the core protein coding sequence, the E1 protein coding sequence, the E2 protein coding sequence, the NS2 protein coding sequence, the NS3 protein coding sequence, the NS4A protein coding sequence, the NS4B protein coding sequence, the NS5A protein coding sequence, the NS5B protein coding sequence, and the 3' untranslated region, in this order in the 5' to 3' direction.

In the more preferable embodiment of this replicon RNA, the genomic RNA of hepatitis C virus of genotype 2a is an RNA comprising a nucleotide sequence shown in SEQ ID NO: 12.

In the still more preferable embodiment of this replicon RNA, the 5' untranslated region comprises a nucleotide sequence shown in SEQ ID NO: 1, the core protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 2, the E1 protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 3, the E2 protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 4, the NS2 protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 5, the NS3 protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 6, the NS4A protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 7, the NS4B protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 8, the NS5A protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 9, the NS5B protein coding sequence comprises a nucleotide sequence shown in SEQ ID NO: 10, and the 3' untranslated region comprises a nucleotide sequence shown in SEQ ID NO: 11.

[2] A replicon RNA, comprising the following RNA (a) or (b):

(a) an RNA comprising a nucleotide sequence shown in SEQ ID NO: 13; or

(b) an RNA comprising a nucleotide sequence derived from the nucleotide sequence shown in SEQ ID NO: 13 by deletion, substitution or addition of 1 to 100 nucleotides, and having autonomous replication ability and virus particle production ability.

[3] A method for producing a cell which replicates a replicon RNA and produces a virus particle, comprising introducing the replicon RNA of any one of [1] or [2] described above

into a cell.

For this method the cell is preferably a proliferative cell. For this method the cell is also or otherwise preferably a eukaryotic cell.

For this method, the eukaryotic cell is preferably a human liver-derived cell, a human uterine cervix-derived cell or a human fetal kidney-derived cell. More preferably, the eukaryotic cell is a Huh7 cell, a HepG2 cell, an IMY-N9 cell, a HeLa cell or a 293 cell.

[4] A cell obtainable by the method of [3] described above, which replicates the replicon RNA and produces the virus particle.

[5] A method for producing a hepatitis C virus particle, comprising culturing the cell of [4] described above to allow the cell to produce the virus particle.

[6] A hepatitis C virus particle obtainable by the method of [5] described above.

[7] A method for producing a hepatitis C virus infected cell, comprising culturing the cell of [4] described above and infecting other cells with the virus particle in the culture.

[8] A hepatitis C virus infected cell obtainable by the method of [7] described above.

[9] A method for screening an anti-hepatitis C virus substance, comprising culturing, in the presence of a test substance, at lease one selected from the group consisting of following (a), (b) and (c):

(a) the cell of [4] described above,

(b) the hepatitis C virus infected cell of [8] described above, and

(c) the hepatitis C virus particle of [6] described above and a hepatitis C virus permissive cell;

and detecting the replicon RNA or the virus particles in the resulting culture.

[10] A hepatitis C vaccine, comprising the hepatitis C virus particle of [6] described above or a part thereof.

[11] A method for producing a hepatitis C vaccine by using the hepatitis C virus particle of [6] described above or part thereof as an antigen.

[12] A method for producing a hepatotropic virus vector for gene therapy by using the replicon RNA of [1] or [2] described above.

[13] A hepatotropic virus vector obtainable by the method of [12] described above.

[14] A method for replicating and/or expressing a foreign gene in a cell, comprising inserting an RNA encoding the foreign gene to the replicon RNA of any one of [1] or [2] described above and introducing it into said cell.

[15] A method for producing a cell which replicates an RNA and produces a virus particle, comprising introducing into the cell the RNA comprising a nucleotide sequence shown in SEQ ID NO. 12.

[16] A method for producing a hepatitis C virus particle, comprising introducing into a cell the RNA comprising a nucleotide sequence shown in SEQ ID NO: 12 and culturing the cell to allow the cell to produce a virus particle.

[17] A method of [15] or [16] described above, wherein the cell is a proliferative cell.

[18] A method for producing a virus vector comprising a foreign gene, comprising inserting an RNA encoding a foreign gene into an RNA comprising the nucleotide sequence shown in SEQ ID NO: 12, introducing it into a cell, and culturing the cell to allow the cell to produce a virus particle.

[19] An antibody against the hepatitis C virus particle of [6] described above

The contents in the description and the drawings of Japanese Patent Application No. 2004-045489, from which the present application claims priority, are incorporated herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic view showing procedures for constructing a template DNA for preparing the full length HCV replicon RNA or the full length HCV genomic RNA of the present invention. The upper part of Figure 1 shows the structure of a plasmid clone pJFH1, which is produced by inserting the full length HCV genome downstream of the T7 promoter. The lower part of Figure 1 shows the structure of plasmid clone pFGREP-JFH1 comprising the full length HCV genomic sequence, in which a DNA fragment containing the neomycin resistance gene and EMCV IRES is inserted downstream of the T7 promoter of pJFH1 and the 5' untranslated region. The terms shown in the Figure are as follows. T7: T7 RNA promoter, 5' UTR: 5' untranslated region, C: core protein, E1, E2: envelope proteins. NS2, NS3, NS4A, NS4B, 4A, 4B: non-structural proteins. 3' UTR: 3' untranslated region. AgeI,

PmeI, XbaI: restriction sites of the restriction enzymes AgeI, PmeI and XbaI. GDD: the site of the amino acids motif GDD which corresponds to the active center of NS5B protein. neo: the neomycin resistant gene. EMCV IRES: encephalomyocarditis virus internal ribosomal entry site;

Figure 2 is a photograph showing the result of a Northern blot analysis demonstrating the replication of rJFH-1 in Huh7 cells to which the full length HCV genomic RNA, rJFH-1, has been introduced;

Figure 3 shows the result of HCV core protein quantitation in the culture medium. The open circle represents cells into which rJFH1 has been introduced, and the closed circle represents cells to which rJFH1/GND has been introduced;

Figure 4 is a graph showing the amounts of HCV core protein and the full length HCV genomic RNA, and the specific gravities for each of fractions that were collected by fractionating of the culture supernatant of rJFH-1-introduced Huh7 cells through sucrose density gradient. The closed circle, open circle and shaded circle represent HCV core protein, the full length HCV genomic RNA and specific gravity, respectively;

Figure 5 is a photograph showing the colony formation of Huh7 cells into which rFGREP-JFH1, the full length HCV replicon RNA, was transfected;

Figure 6 is a photograph showing the replication of full length HCV replicon RNA in the full length HCV replicon RNA-replicating cell clone, which has been established by transfecting rFGREP-JFH1 into Huh7 cells;

Figure 7 is a photograph showing the result of PCR amplification using the genomic DNA of the host cell as a template and the primers specific for the neomycin resistant gene, for confirming the integration of the neomycin resistance gene into the genomic DNA. M: DNA size marker, P: Positive control, N: Huh7 cells;

Figure 8 is a photograph showing the result of a Western blotting analysis demonstrating the expression of core protein in Huh7 cells into which rFGREP-JFH1, the full length HCV replicon RNA, has been introduced;

Figure 9 is a photograph showing the result of a Western blotting analysis demonstrating the expression of NS3 protein in Huh7 cells into which rFGREP-JFH1, the full

length HCV replicon RNA, has been introduced;

Figure 10 is a photograph showing the result of a Western blotting analysis demonstrating the expression of NS5A protein in Huh7 cells into which rFGREP-JFH1, the full length HCV replicon RNA, has been introduced;

Figure 11 is a graph showing the amounts of HCV core protein and full length HCV replicon RNA, and the specific gravities for each of fractions that were collected by fractionating of the culture supernatant of rFGREP-JFH1-introduced Huh7 cells through sucrose density gradient. The closed circle, open circle and shaded circle represent HCV core protein, the full length HCV replicon RNA and specific gravity, respectively; and

Figure 12 is a photograph showing the colony formation of Huh7 cells to which virus particles in the culture supernatant of the full length HCV replicon RNA-replicating cell have been added.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is explained in detail as follows.

1. Full length HCV replicon RNA

The genome of hepatitis C virus (HCV) is a single-stranded (+) strand RNA comprising approximately 9600 nucleotides. This genomic RNA comprises the 5' untranslated region (also denoted as 5' NTR or 5' UTR), a translated region composed of a structural region and a non-structural region, and the 3' untranslated region (also denoted as 3' NTR or 3' UTR). HCV structural proteins are encoded in the structural region, and a plurality of non-structural proteins are encoded in the non-structural region.

Such HCV structural proteins (core, E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) are generated by first translating the translated region into a single continuous polyprotein and then releasing by having restricted cleavage of the polyprotein by proteases. Among these structural proteins and non-structural proteins (that is, viral proteins of HCV), core is a core protein, E1 and E2 are envelope proteins. The non-structural proteins are proteins involved in viral own replication, and NS2 is known to have metalloprotease activity, and NS3 is known to have serine protease activity (at one-third

of the N terminal side) and helicase activity (at two-thirds of the C-terminal side). Furthermore, NS4A is a cofactor for protease activity of NS3, and NS5B has been reported to have RNA-dependent RNA polymerase activity.

The present inventors constructed a replicon RNA having autonomous replication ability and virus particles production ability, using HCV genomic RNA.

RNA having autonomous replication ability which has been produced by modifying the HCV genomic RNA is called "replicon RNA" or "RNA replicon" herein. In the present specification, the replicon RNA derived from HCV may also be called HCV-RNA replicon. The replicon RNA of the present invention comprising the full length of HCV genomic RNA is called "full length HCV replicon RNA" herein. The full length HCV replicon RNA of the present invention has an ability of producing virus particles.

In the preferred embodiment of the full length HCV replicon RNA in the present invention, hepatitis C virus is, but not limited to, preferably hepatitis C virus of genotype 2a. In the present invention, "hepatitis C virus of genotype 2a" or "HCV of genotype 2a" means a hepatitis virus identified as the genotype 2a according to the international classification by Simmonds et al. (see Simmonds, P. et al, Hepatology, (1994) 10, p. 1321-1324). In the present invention, "hepatitis C virus of genotype 2a" or "HCV of genotype 2a" includes not only virus having naturally-occurring HCV genomic RNA but also virus having a genomic RNA in which the naturally-occurring HCV genomic sequence is modified artificially. A particular example of the HCV of genotype 2a includes JFH-1 strain (see JP Patent Publication (Kokai) No. 2002-171978)

In the present specification, "the genomic RNA of hepatitis C virus" means RNA comprising the nucleotide sequence over the entire region of the single-stranded (+) sense RNA genome of hepatitis C virus . The genomic RNA of hepatitis C virus of genotype 2a is, but not limited to, preferably RNA comprising the nucleotide sequence shown in SEQ ID NO: 12.

One of the embodiments of the full length HCV replicon RNA according to the present invention is a replicon RNA comprising the nucleotide sequence comprising a 5' untranslated region, a core protein coding sequence, an E1 protein coding sequence, an E2

protein coding sequence, an NS2 protein coding sequence, an NS3 protein coding sequence, an NS4A protein coding sequence, an NS4B protein coding sequence, an NS5A protein coding sequence, an NS5B protein coding sequence, and a 3' untranslated region, at least one selectable marker gene or reporter gene, and at least one IRES sequence.

It is not limited but preferable that the full length HCV replicon RNA according to the present invention comprises: the 5' untranslated region, at least one selectable marker gene or reporter gene, at least one IRES sequence, the core protein coding sequence, the E1 protein coding sequence, the E2 protein coding sequence, the NS2 protein coding sequence, the NS3 protein coding sequence, the NS4A protein coding sequence, the NS4B protein coding sequence, the NS5A protein coding sequence, the NS5B protein coding sequence, and the 3' untranslated region, in this order in the 5' to 3' direction.

In the specification of the present application, "5' untranslated region" (5' NTR or 5' UTR), "core protein coding sequence" (core region or C region), "E1 protein coding sequence" (E1 region), "E2 protein coding sequence" (E2 region), "NS2 protein coding sequence" (NS2 region), "NS3 protein coding sequence" (NS3 region), "NS4A protein coding sequence" (NS4A region), "NS4B protein coding sequence" (NS4B region), "NS5A protein coding sequence" (NS5A region), "NS5B protein coding sequence" (NS5B region) and "3' untranslated region" (3' NTR or 3' UTR), and other specific regions or sites are defined based on the full length genomic RNA (SEQ ID NO: 12) comprising the entire region of the genome of the JFH-1 strain (JP Patent Publication (Kokai) No. 2002-171978), which is a HCV virus of genotype 2a.

Also, a partial region or site in the genome of hepatitis C virus (HCV) according to the present invention may be defined based on the sequences shown in SEQ ID NOs: 1-11 that are the partial nucleotide sequences of the genomic RNA of JFH-1 strain (SEQ ID NO: 12). "5' untranslated region" of the full length genomic RNA of JFH-1 strain (derived from JFH-1 clone; SEQ ID NO: 12) comprises the nucleotide sequence shown in SEQ ID NO: 1. "Core protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 2. "E1 protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 3. "E2 protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 4. "NS2

protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 5. "NS3 protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 6. "NS4A protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 7. "NS4B protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 8. "NS5A protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 9. "NS5B protein coding sequence" comprises the nucleotide sequence shown in SEQ ID NO: 10. "3' untranslated region" comprises the nucleotide sequence shown in SEQ ID NO: 11.

For example, a region or site in the RNA sequence derived from HCV may be defined by the nucleotide numbers within the nucleotide sequences of SEQ ID NOs. 1-12 which are determined by alignment of the RNA sequence and the nucleotide sequences shown in the SEQ ID NOs. 1-12. In the alignment, a gap, addition, deletion, substitution and the like may be present.

In more preferable embodiment of the present invention, the 5' untranslated region, the core protein coding sequence, the E1 protein coding sequence, the E2 protein coding sequence, the NS2 protein coding sequence, the NS3 protein coding sequence, the NS4A protein coding sequence, the NS4B protein coding sequence, the NS5A protein coding sequence, the NS5B protein coding sequence, and the 3' untranslated region , which are contained in the full length HCV replicon RNA, preferably comprises the nucleotide sequences shown in SEQ ID NOs. 1-11, respectively.

A preferred embodiment of the full length HCV replicon RNA according to the present invention is a replicon RNA comprising nucleotide sequences shown in SEQ ID NOs: 1-11, at least one marker gene and/or reporter gene, and at lease one IRES sequence.

"Selectable marker gene" in the present invention means a gene conferring selectability to a cell so that only the cell expressing the gene can be selected. A general example of the selectable marker gene includes an antibiotic resistant gene. The examples of the selectable marker gene preferred in the present invention include a neomycin resistance gene, a thymidine kinase gene, a kanamycin resistance gene, a pyrithiamine resistance gene, an adenylyl transferase gene, a Zeocin resistance gene and a puromycin resistance gene. The neomycin resistance gene and the thymidine kinase gene are preferred, and the neomycin

resistance gene is more preferred. However, the selectable marker gene in the present invention is not limited to these genes.

Furthermore in the present invention, "reporter gene" means a marker gene encoding a gene product that may act as an indicator for the expression of the gene. General examples of a reporter gene include structural genes of enzymes that catalyze light emitting reaction or color reaction. Preferred examples of the reporter gene in the present invention include transposon Tn9-derived chloramphenicol acetyltransferase gene, Escherichia coli-derived β-glucuronidase gene or β-galactosidase gene, luciferase gene, a green fluorescent protein gene, aequorin gene from jellyfish, and secreted placental alkaline phosphatase (SEAP) gene. However, the reporter gene in the present invention is not limited to these genes.

Either only one or both of the above selectable marker gene and reporter gene may be contained in a full length replicon RNA. One or more of the selectable marker genes or reporter genes may be present in one full length HCV replicon RNA.

In the present invention, "IRES sequence" means an internal ribosome entry site that allows translation to be initiated by binding ribosomes within the inside of the RNA. Preferred examples of IRES sequence in the present invention include, but are not limited to, EMCV IRES (the internal ribosome entry site of encephalomyocarditis virus), FMDV IRES and HCV IRES. EMCV IRES and HCV IRES are more preferred, and EMCV IRES is the most preferred sequence.

A still more preferred embodiment of a full length HCV replicon RNA according to the present invention is an RNA comprising the nucleotide sequence shown in SEQ ID NO: 13. Furthermore, a replicon RNA comprising a nucleotide sequence derived from the nucleotide sequence shown in SEQ ID NO: 13 by deletion, substitution or addition of 1-100, preferably 1-30, more preferably 1-10, still more preferably 1-6 and most preferably one to several (2-5) nucleotides in the nucleotide sequence shown in SEQ ID NO: 13 and having autonomous replication ability and virus particle production ability is a preferred embodiment of the full length HCV replicon RNA and also included in the scope of the present invention .

The full length HCV replicon RNA according to the present invention may also contain an RNA encoding an optional foreign gene to be expressed within a cell into which the

full length replicon RNA is introduced. The RNA encoding the foreign gene may also be ligated downstream of the 5' untranslated region or ligated upstream or downstream of a selectable marker gene or a reporter gene, or ligated upstream of the 3' untranslated region. The RNA encoding the foreign gene may be inserted in any site between the core protein coding sequence, the E1 protein coding sequence, the E2 protein coding sequence, the NS2 protein coding sequence, the NS3 protein coding sequence, the NS4A protein coding sequence, the NS4B protein coding sequence, the NS5A protein coding sequence and the NS5B protein coding sequence.

The full length HCV replicon RNA containing the RNA encoding the foreign gene can express a gene product encoded by the foreign gene when it is translated within a cell into which the RNA is introduced. Thus, the full length HCV replicon RNA containing the RNA encoding the foreign gene can be also appropriately used for producing a gene product from the foreign gene within a cell.

The full length HCV replicon RNA according to the present invention may further contain a ribozyme. A ribozyme is ligated downstream of a selectable marker gene and/or a reporter gene so that the selectable marker gene and/or the reporter gene may be cut off by the self cleavage activity of a ribozyme from the IRES sequence, the core protein coding sequence, the E1 protein coding sequence, the E2 protein coding sequence, the NS2 protein coding sequence, the NS3 protein coding sequence, the NS4A protein coding sequence, the NS4B protein coding sequence, the NS5A protein coding sequence and the NS5B protein coding sequence, and the 3' untranslated region.

In the full length HCV replicon RNA according to the present invention, the above described selectable marker gene and/or reporter gene, the sequences encoding viral proteins, and the foreign gene, ribozyme or the like are ligated so that they are translated from the full length HCV replicon RNA in the correct reading frame. Among these sequences, the proteins encoded by the full length replicon RNA are preferably connected to each other via protease cleavage sites and the like, so that the proteins are translated or expressed as a polyprotein, followed by cleaving by protease into each protein.

The present invention also relates to a DNA vector, preferably an expression vector,

which encodes the replicon RNA of the present invention.

In the present invention "autonomous replication ability" of RNA means that the RNA is capable of growing autonomously when introduced into the cell. The autonomous replication ability of RNA may be confirmed by the following procedure although it is not limited. Huh7 cells are transfected with the RNA of interest and cultured. RNAs are extracted from the resulting cultured cells and subjected to Northern blot hybridization using a probe capable of specifically detecting the introduced RNA. Detection of the RNA of interest confirms the autonomous replication. Examples of the particular procedure for confirming the autonomous replication ability are illustrated in the descriptions about assay of colony forming ability, confirmation of HCV protein expression, detection of replicon RNA and the like in the Examples of the present specification.

Further, in the present invention, "virus particle production ability" of RNA means that virus particles are generated in a cell when the RNA is introduced into the cell (e.g. cultured cell such as Huh7 cells). The virus particle production ability may be confirmed, for example, by applying for detection the RT-PCR method using primers specific to the RNA to the culture supernatant of the RNA-introduced cell. It may also be confirmed by subjecting the culture supernatant to the sucrose density gradient method to separate virus particles and by detecting HCV protein. Examples of the particular procedure are illustrated in the descriptions about assay of colony forming ability, confirmation of HCV protein expression, detection of replicon RNA and the like in the Examples of the present specification.

2. Preparation of full length HCV replicon RNA

The full length HCV replicon RNA according to the present invention can be prepared using genetic engineering techniques known to persons skilled in the art. The full length HCV replicon RNA may be prepared, but not limited to, for example, using JFH-1 strain as hepatitis C virus of genotype 2a by the following method.

First, DNA corresponding to the entire region of the genomic RNA of JFH-1 strain (SEQ ID NO: 12; this sequence is registered at international DNA data bank under accession No. AB047639) is routinely reconstructed and inserted downstream of an RNA promoter so as

to prepare a DNA clone. As used herein, "DNA corresponding to RNA" means a DNA having a nucleotide sequence derived from the nucleotide sequence of the RNA by substituting U (uracil) with T (thymine). The above RNA promoter is preferably contained in a plasmid clone. An example of the preferred RNA promoter is not limited to, but includes T7 RNA promoter, SP6 RNA promoter and SP3 RNA promoter, and T7 RNA promoter is particularly preferred.

Next, the selectable marker gene and/or reporter gene, and DNA encoding the IRES sequence are inserted into the DNA clone described above. It is preferred to insert the selectable marker gene and/or reporter gene downstream of 5' untranslated region and the IRES sequence further downstream.

Subsequently, using the DNA clone prepared as above as a template, RNA is synthesized using RNA polymerase. RNA synthesis can be initiated by a standard procedure from the 5' untranslated region. When the DNA clone is a plasmid clone, RNA can be synthesized using the DNA fragment excised from the plasmid clone with a restriction enzyme, as a template. In addition, it is preferable that the 3' terminus of RNA to be synthesized has the same sequence as the terminus of the 3' untranslated region of the viral genomic RNA, and no other sequences are added or deleted. The thus synthesized RNA is the full length HCV replicon RNA according to the present invention.

3. Preparation of HCV particles

A recombinant cell that can replicate the full length HCV replicon RNA, preferably continuously replicate (i.e., which has a replicon RNA-replication ability), can be obtained by introducing the full length HCV replicon RNA prepared as described above into a cell. In this specification, a recombinant cell that replicates the full length HCV replicon RNA is referred to as a "full length HCV replicon RNA-replicating cell."

The full length HCV replicon RNA-replicating cell can produce virus particles. The produced virus particles contain the full length HCV replicon RNA in a shell composed of HCV virus proteins. Thus, the virus particles produced by the full length HCV replicon RNA-replicating cell of the present invention are HCV particles. That is, in the present

invention, HCV particles can be prepared in a cell culture system by culturing the full length HCV replicon RNA-replicating cells. Preferably, HCV particles can be obtained by culturing the full length HCV replicon RNA-replicating cells and collecting the virus particles generated in the culture (preferably the culture supernatant).

Alternatively, HCV particles can be produced by a recombinant cell which is obtained by introducing the full length HCV genomic RNA into a cell. The full length HCV genomic RNA is replicated with high efficiency in the cell, into which the full length HCV genomic RNA of the present invention (preferably the full length HCV genomic RNA derived from JFH-1 clone, and more preferably RNA having the nucleotide sequence shown in SEQ ID NO: 12) is introduced. In this specification, a cell that replicates the full length HCV genomic RNA is referred to as a "full length HCV genomic RNA-replicating cell". The full length HCV genomic RNA-replicating cells can produce virus particles. The virus particles produced by the full length HCV genomic RNA-replicating cells contain the full length HCV genomic RNA in a shell composed of HCV virus proteins. Thus, the virus particles produced by the cell into which the full length HCV genomic RNA of the present invention is introduced are HCV particles. It is not limited but preferred that HCV particles may be prepared in a cell culture system by culturing the cell into which the full length HCV genomic RNA derived from JFH-1 clone (e.g. RNA having the nucloetide sequence shown in SEQ ID NO: 12) is introduced. For example, HCV particles can be obtained by culturing the cells into which the full length HCV genomic RNA (e.g. RNA having the nucleotide sequence shown in SEQ ID NO: 12) is introduced and collecting virus particles generated in the culture (preferably the culture supernatant).

For a cell into which the full length HCV replicon RNA or the full length HCV genomic RNA described above is to be introduced, any cell can be used, as long as it can be subcultured. Such a cell is preferably a eukaryotic cell, more preferably a human cell, and still more preferably a human liver-derived cell, a human uterine cervix-derived cell or a human fetal kidney-derived cell. Proliferative cells including cancer cell lines, stem cell lines and the like cells can be used preferably, and Huh7 cells, HepG2 cells, IMY-N9 cells, HeLa cells and 293 cells and the like are used more preferably. For these cells, commercially

available cells may be utilized, these cells may be obtained from cell depositories, or cell lines established from any cells (e.g., cancer cells or stem cells) may also be used.

Introduction of the full length HCV replicon RNA or the full length HCV genomic RNA into cells can be achieved using any technique known to persons skilled in the art. Examples of such an introduction method include electroporation, particle gun method, lipofection method, calcium phosphate method, microinjection method, DEAE sepharose method and the like. The method using electroporation is particularly preferred.

The full length HCV replicon RNA or the full length HCV genomic RNA may be introduced alone, or may be introduced after being mixed with other nucleic acids. To vary the amount of the full length HCV replicon RNA or the full length HCV genomic RNA while keeping RNA amount to be introduced at a certain level, the desired amount of the full length HCV replicon RNA or the full length HCV genomic RNA to be introduced is mixed with total cellular RNA extracted from the cells, to which the RNA is introduced, to bring the total RNA amount up to a certain level, and then the mixture is used for introduction into cells. The amount of replicon RNA to be used for introducing into cells may be determined according to the introduction method employed, and is preferably between 1 picogram and 100 micrograms, and more preferably between 10 picograms and 10 micrograms.

The full length HCV replicon RNA-replicating cells can be selected utilizing the expression of the selectable marker gene or the reporter gene within the full length HCV replicon RNA. Specifically, for example, such cells subjected to the treatment for cellular introduction of the full length HCV replicon RNA may be cultured in a medium, in which the cells can be selected due to the expression of the selectable marker gene. Alternatively, after culturing the cells subjected to the treatment for cellular introduction of the full length HCV replicon RNA, the expression of the reporter gene (for example fluorescent protein) may be detected.

As an example, when the full length HCV replicon RNA contains a neomycin resistance gene as a selectable marker gene, cells subjected to electroporation method with the full length HCV replicon RNA, are seeded into a culture dish. After culturing 12 to 72 hours, preferably 16 to 48 hours, G418 (neomycin) is added to the culture dish at a concentration of

0.05 milligrams/milliliter to 3.0 milligrams/milliliter. The cells are continuously cultured for preferably 10 days to 40 days and more preferably 14 days to 28 days after seeding, while changing the culture medium twice a week, and the cells that is replicating the introduced full length HCV replicon RNA, can be selected as a colony by staining viable cells with crystal violet.

Cells can be cloned from the formed colonies by standard procedure. The thus obtained cell clone that replicates the full length HCV replicon RNA is referred to as "a full length HCV replicon RNA-replicating cell clone" in this specification. The full length HCV replicon RNA-replicating cell of the present invention includes the full length HCV replicon RNA-replicating cell clone.

For the full length HCV replicon RNA-replicating cell, actual replication of the full length HCV replicon RNA in the cell or cell clone can be confirmed by detecting the replicated full length HCV replicon RNA, confirming that the selectable marker or reporter gene of the full length HCV replicon RNA is not integrated in the host genomic DNA and further detecting HCV proteins.

The full length HCV replicon RNA that has been replicated may be detected according to any RNA detection method known to persons skilled in the art. For example, the full length HCV replicon RNA can be detected in total RNA extracted from the cell by the Northern hybridization method using a DNA fragment specific to the full length HCV replicon RNA as a probe.

Furthermore, the absence of the integrated selectable marker gene or reporter gene in the full length HCV replicon RNA in the host genomic DNA can be confirmed by, but not limited to, for example, performing PCR for the genomic DNA extracted from the cell to amplify at least a part of the selectable marker gene or reporter gene, and then confirming the absence of the amplified product. Since it is considered that in the cell, for which the amplified product is confirmed, the selectable marker gene or reporter gene may have been integrated in the host genome, it is possible that the full length HCV replicon RNA itself is not replicated. In this case, the replication of the full length HCV replicon RNA can be further confirmed by detecting HCV proteins as described below.

An HCV protein can be detected by, for example, reacting an antibody against the HCV protein to be expressed from the introduced full length HCV replicon RNA with the extracted cellular proteins. This method can be carried out by any protein detection method known to persons skilled in the art. Specifically, HCV protein can be detected by, for example, blotting a protein sample extracted from the cell onto a nitrocellulose membrane, reacting an anti-HCV protein antibody (e.g., anti-NS3 specific antibody or antiserum collected from a hepatitis C patient) with the nitrocellulose membrane and detecting the anti-HCV protein antibody. If the HCV protein is detected among the extracted cellular proteins, it can be concluded that this cell replicates the full length HCV replicon RNA and expresses the HCV protein.

The virus particle production ability of the full length HCV replicon RNA-replicating cells or the full length HCV genomic RNA-replicating cells may be confirmed by any virus detection method known to the persons skilled in the art. For example, the culture supernatant of cells which are suspected of producing virus particles is fractionated through the sucrose density gradient, and the density of fraction, HCV core protein concentration, and amount of the full length HCV replicon RNA or the full length HCV genomic RNA are determined for each fraction. As a result, if the peak of the core protein coincides with that of the full length HCV replicon RNA or the full length HCV genomic RNA, and the density of the fraction showing the detected peaks (e.g. 1.18-1.20 mg) is smaller than the density of the equivalent fraction as obtained by fractionating the culture supernatant treated with 25% NP40 (Polyoxyethylene(9)Octylphenyl Ether), the cells can be considered to have a virus particle production ability.

HCV virus particles released in the culture supernatant can be detected, for example, using antibodies to the core protein, the E1 protein or the E2 protein. Also, the presence of HCV virus particles can be detected indirectly by amplifying and detecting the full length HCV replicon RNA in the culture supernatant by the RT-PCR method using specific primers.

4. Infection of another cell with HCV particles of the present invention

HCV virus particles of the present invention have an ability to infect a cell

(preferably an HCV permissive cell). The present invention relates also to a method for producing a hepatitis C virus-infected cell comprising culturing the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cell, and infecting another cell (preferably an HCV permissive cell) with virus particles in the thus obtained culture (preferably culture supernatant). In the present invention, the HCV permissive cell means a cell which is susceptible to HCV, and is preferably, but not limited to, a hepatic cell or a lymphoid lineage cell. In particular, the hepatic cell includes a primary hepatocyte, Huh7 cell, HepG2 cell, IMY-N9 cell, HeLa cell, 203 cell and the like. The lymphoid lineage cell includes, but not limited to, Molt4 cell, HPB-Ma cell, Daudi cell and the like.

When a cell (e.g., an HCV permissive cell) is infected with HCV particles produced by the full length HCV replicon RNA-replicating cell of the present invention, the full length HCV replicon RNA is replicated and virus particles are also formed in the infected cell. Since the cell infected with virus particles generated in the full length HCV replicon RNA-replicating cell expresses the selectable marker gene and/or reporter gene, the infected cell can be selected and/or detected by utilizing the expression. By infecting a cell with virus particles generated in the full length HCV replicon RNA-replicating cell of the present invention, the full length HCV replicon RNA is replicated in the cell and furthermore the virus particles can be produced.

Still further, by infecting a cell (e.g. an HCV permissive cell) with HCV particles generated in the full length HCV genomic RNA-replicating cell of the present invention, the full length HCV genomic RNA is replicated and virus particles are also formed in the infected cell. By infecting a cell with virus particles generated in the full length HCV genomic RNA-replicating cell of the present invention, the full length HCV genomic RNA is replicatedin the cell and furthermore the virus particles can be produced.

HCV virus particles generated in the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cell can infect HCV permissive animals such as chimpanzee and the like and induce hepatitis caused by HCV therein.

5. Other embodiments of the present invention

The full length HCV replicon RNA is replicated with a high efficiency in the full length HCV replicon RNA-replicating cell of the present invention. Also the full length HCV genomic RNA is replicated with a high efficiency in the full length HCV genomic RNA-replicating cell of the present invention. Thus, the full length HCV replicon RNA or the full length HCV genomic RNA can be produced with a high efficiency using the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cell of the present invention.

In the present invention the full length HCV replicon RNA can be produced by culturing the full length HCV replicon RNA-replicating cell, extracting RNA from the culture (cultured cells and/or culture medium), subjecting the RNA to the electrophoresis method, and isolating and purifying the full length HCV replicon RNA. The full length HCV genomic RNA can also be produced by using the full length HCV genomic RNA-replicating cell by the similar method. The RNA produced by such a way comprises the full length genomic sequence of hepatitis C virus. In this case the full length genomic sequence of hepatitis C virus may be interrupted by the selectable marker gene and/or reporter gene and the IRES sequence. By the method for producing the RNA comprising the full length genomic sequence of hepatitis C virus being provided, more detailed analysis of hepatitis C virus genome becomes possible.

Further, the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cell of the present invention can be suitably used for producing HCV protein. HCV protein may be produced by any method known to persons skilled in the art. For example, HCV protein may be produced by introducing the full length HCV replicon RNA or the full length HCV genomic RNA into a cell, culturing the recombinant cell and collecting proteins from the culture thus obtained (cultured cells and/or culture medium) by the known procedure.

Further, the HCV virus particles of the present invention may possess hepatotropism. Thus a hepatotropic virus vector can be produced using the full length HCV replicon RNA of the present invention. This virus vector is suitably used for gene therapy. In the present invention, a foreign gene can be introduced into a cell, replicated in the cell and expressed, by

integrating an RNA encoding the foreign gene into the full length HCV replicon RNA or full length HCV genomic RNA and introducing the integrated RNA into the cell. Further, by preparing an RNA in which the E1 protein coding sequence and/or the E2 protein coding sequence of the full length HCV replicon RNA or full length HCV genomic RNA are replaced with an outer shell protein coding sequence of virus derived from other biological species, it becomes possible to infect the RNA to various biological species. In this case also, a foreign gene is integrated into the full length HCV replicon RNA or full length HCV genomic RNA and this can be used as a hepatotropic virus vector for expressing the foreign gene in hepatocytes.

The present invention relates also to a method for producing a virus vector carrying a foreign gene, comprising inserting an RNA encoding the foreign gene into RNA comprising the nucleotide sequence shown in SEQ ID NO: 12, introducing it into a cell and culturing the cell to produce virus particles.

The present invention provides a hepatitis C vaccine comprising HCV particles of the present invention or a part thereof and a method for producing the hepatitis C vaccine comprising HCV particles of the present invention or a part thereof.

In particular, HCV particles as prepared above may be used directly as a vaccine or may be used after attenuating or inactivating by the known method in the art. For example, a HCV vaccine stock solution can be obtained by purifying the HCV particles using column chromatography, filtration, centrifugation and the like. An attenuated live HCV vaccine or an inactivated HCV vaccine may be prepared from this HCV vaccine stock solution. Inactivation of virus can be carried out by reacting an inactivation agent such as formalin, β-propiolactone, glutardialdehyde and the like with the virus, by adding and mixing to, for example, virus suspension (Appaiahgari et al., Vaccine, (2004) 22(27-28), p.3669-3675).

For the production of the vaccine of the present invention, it is possible to use HCV replicon RNA in which the pathogenicity is attenuated or lost by an introduced mutation using the publicly known art.

The vaccine of the present invention is prepared for administration as a solution or suspension. It is also possible to be prepared in the form of solid material suitable for

dissolving or suspending in liquid. The preparation may be emulsified or capsulized in liposome. The active immunogenic component such as HCV particles is often mixed with an excipient which is pharmaceutically acceptable and appropriate for the active ingredient. A suitable excipient includes, for example, water, physiological saline, dextrose, glycerol, ethanol and mixtures thereof. Further, if desired, the vaccine may contain a small amount of auxiliary agent (e.g. humidifier or emulsifier), pH buffer and/or adjuvant for enhancing the efficacy of the vaccine. Examples of effective adjuvant include but not limited to following substances: aluminum hydroxide, N-acetyl-muramyl- L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl- D-isoglutamine (CGP11637, nor-MDP), N-acetyl muramyl-L-alanyl- D-isoglutaminyl- L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphryloxy)-ethylamine (CGP19835A, referred to as MTP-PE) and RIBI. RIBI contains three components extracted from bacteria, that is monophosphoryl lipid A, trehalose dimycolate and cell wall skelton (HPL + TDM + CWS), in 2% squalene/Tween$^{(R)}$ 80 emulsion. Efficacy of an adjuvant can be determined by measuring the amount of antibody against the immunogenic HCV particles which is produced by administrating the vaccine composed of HCV particles.

The present vaccine is normally administered parenterally, for example by injection such as subcutaneous or intramuscular injection. Other dosage forms suitable for the other administration route include suppository and, in some case, oral formulation.

If desired, one or more of the above compounds having adjuvant activity may be added to the HCV vaccine. The adjuvants are a non-specific stimulating factor for this immune system and enhance the immune response to HCV vaccine in the host. Particular examples of adjuvant known in this technical art include complete Freund's adjuvant, incomplete Freund's adjuvant, vitamin E, nonionic block polymer, muramyldipeptide, saponin, mineral oil, vegetable oil and Carbopol. Adjuvants especially suitable for application for the mucosal membrane include, for example, E. coli heat labile toxin (LT) and cholera toxin (CT). Other suitable adjuvants include, for example, aluminum hydroxide, aluminum phosphate or aluminum oxide, oil emulsion (e.g. Bayol (Registered Trade Mark) or Marcol 52 (Registered Trade Mark)), saponin or vitamin E solubilisate. In the preferred embodiment, the vaccine of

the present invention contains an adjuvant.

For examples, for the injections to be administered subcutaneously, intradermally, intramuscularly and intravenously, particular examples of pharmaceutically acceptable carriers and diluents, which can be included in the HCV vaccine of the present invention, include stabilizers, carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein-containing materials such as bovine serum albumin or skim milk, and buffers (e.g. phosphate buffer).

Conventional binders and carriers used for a suppository include, for example, polyalkyleneglycol or triglycerides. The suppository can be formulated from a mixture containing the active ingredient in the range of 0.5% to 50%, preferably 1% to 20%. An oral formulation may contain normally used excipients. Such excipients include, for example, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like of pharmaceutical grade.

The vaccine of the present invention can be produced in the dosage forms of solutions, suspensions, tablets, pills, capsules, extended release formulations or powders and contain the active ingredient (virus particles or a part thereof) at 10-95%, preferably 25-70%.

The vaccine of the present invention is administered by the method suitable for the dosage forms and at the effective amount for prevention and/or treatment. The dosage amount is in the range from 0.01 μg to 100,000 μg and this is dependent on the patient to be treated, the antibody forming capability in the immune system of the patient, and desired level of protection. It is also dependent of the administration route such as oral, subcutaneous, intradermal, intramuscular, intravenous and the like.

This vaccine may be administered by the single administration schedule or preferably by the complex administration schedule. In the complex administration schedule, 1-10 individual administrations are carried out at the start of administration, followed by administrations at intervals required to sustain and/or to enhance the immune response. For example, another type of administration may be given as the second administration 1-4 months later. If necessary, the administration may be continued several months later. The administration regimen is, at least partially, determined according to the need for the

individual patient and is dependent on the judgment of the attending physician.

Further, the vaccine containing immunogenic HCV particles may be co-administered with other immune controlling agent (e.g. immunoglobulin).

The HCV particle vaccine can be used preventively against the possible new HCV infection by administering to healthy individuals to induce the immune response to HCV. The HCV particle vaccine can also be used as a therapeutic vaccine to eliminate HCV by administering to patients infected with HCV and inducing a strong immune response to HCV in the body.

The full length HCV replicon RNA-replicating cell or full length HCV genomic RNA-replicating cell or the hepatitis C virus-infected cell, which is infected with virus particles generated in these cells, can be used as a test system for screening a substance (anti-hepatitis C virus substance) which promotes or inhibits, for example, the replication of hepatitis C virus, re-construction of virus particles and release of virus particles. In particular, for example, the substance which promotes or inhibits the growth of hepatitis C virus can be screened by determining whether the test substance promotes or inhibits the replication of the full length HCV replicon RNA or the full length HCV genomic RNA, or formation or release of the virus particles, culturing these cells in the presence of the test substance and detecting the full length HCV replicon RNA or the full length HCV genomic RNA, or the virus particles in the obtained culture. In this case, the detection of the full length HCV replicon RNA or the full length HCV genomic RNA in the culture may be carried out by determining the amount, the ratio or the presence of the full length HCV replicon RNA or the full length HCV genomic RNA in the RNA preparation extracted from cells described above. The detection of the virus particles in the culture (mainly culture supernatant) may be carried out by measuring the amount, the ratio or the presence of HCV protein in the culture supernatant.

Furthermore, it can be investigated whether immunoglobulin purified from the serum of a HCV infected patient can prevent the infection with HCV particles of the present invention, by detecting virus particles in this culture. In this test, sera from mice, rats, rabbits and the like, which has been immunized with the HCV virus particles of the present invention, can be used. Immunization by a part of HCV protein, the HCV gene and the like may be

utilized. This test may be performed on the other infection preventive substances in a similar manner.

The antibodies of the present invention which are generated against HCV virus particle of the present invention include polyclonal antibodies and monoclonal antibodies. When the polyclonal antibody is preferred, selected mammals (e.g. mouse, rabbit, goat, sheep, horse and the like) are immunized with the HCV particles of the present invention as the first step. Sera are collected from immunized animals and processed by the known procedure. If the sera containing polyclonal antibodies to HCV epitopes contain antibodies to other antigens, these sera may be purified by immunoaffinity chromatography. The methods for generating polyclonal antisera and the methods for treatment of it are known in the art. Polyclonal antibodies may be isolated from mammals already infected with HCV.

Monoclonal antibodies to HCV epitopes can be produced easily by persons skilled in the art. The common method for producing hybridoma which generates monoclonal antibodies is known. For example, the methods described in Current Protocols in Immunology (John Wiley & Sons, Inc.) can be used.

The monoclonal antibody-generating cell lines may be produced by cell fusion, or by other method such as direct transformation of B lymphocyte with tumor gene DNA or transduction with Epstein-Barr virus.

Monoclonal antibodies and polyclonal antibodies obtained by these methods are useful for diagnosis, treatment and prevention of HCV.

The antibodies produced by using the HCV particles of the present invention are administered with pharmaceutically acceptable solubilizer, additive, stabilizer, buffer and the like. Any administration route can be chosen but subcutaneous, intradermal and intramuscular administrations are preferred and intravenous administration is more preferred.

The HCV particles, generated in the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cell of the present invention, and HCV permissive cell can be used as a test system for screening a substance which may stimulate or inhibit the binding of HCV to cells. In particular, for example, substances, which may promote or inhibit the growth of hepatitis C virus, can be screened by culturing the HCV

particles generated in the full length HCV replicon RNA-replicating cell of the present invention together with HCV permissive cell in the presence of a test substance, detecting the full length HCV replicon RNA or virus particles in the culture obtained and determining whether the test substance promotes or inhibits the replication of the replicon RNA or formation of virus particles

Such detections of full length HCV replicon RNA or full length HCV genomic RNA, or virus particles can be carried out according to the technique described above or following Examples. The test system described above can be used for the production and evaluation of the preventive, therapeutic or diagnostic agents of hepatitis C virus infection.

In particular, examples of the usage of the test system of the present invention described above include following:

(1) Screening for a substance which inhibits growth and infection of HCV

The substances which inhibit growth and infection of HCV include, for example, organic compounds which affect the growth and infection of HCV directly or indirectly, anti-sense oligonucleotide or the like which affect the growth of HCV or translation of HCV protein directly or indirectly by hybridizing with the target sequence in the HCV genome or its complementary strand.

(2) Evaluation of various substances which have antivirus activity in cell culture.

The aforementioned various substances include substances obtained by rational drug design or high-throughput screening (for example, purified and isolated enzyme).

(3) Identification of a new target for the treatment of patients infected with HCV

For example, the full length HCV replicon RNA-replicating cell or the full length HCV genomic RNA-replicating cells of the present invention can be used for identifying host cellular protein which may play an important role for the growth of HCV

(4) Evaluation of the ability of HCV for acquiring resistance to drugs and the like, and identification of the mutation related to the resistance

(5) Production of virus protein as an antigen usable for development, production and evaluation of diagnostic and therapeutic agents for hepatitis C virus infection

(6) Production of virus protein as an antigen usable for development, production and

evaluation of the vaccine for hepatitis C virus infection and production of attenuated HCV

(7) Production of monoclonal or polyclonal antibodies for diagnosis and treatment of hepatitis C virus infection.

The present invention will be described more specifically based on the following examples and drawings. However, the technical scope of the present invention is not limited to these examples.

Example 1: Preparation of the full length HCV replicon RNA derived from the full length HCV genomic RNA

(A) Construction of expression vector

Plasmid DNAs were constructed in which DNAs (JFH-1 clone) containing the full length genomic cDNA of hepatitis C virus JFH-1 strain (genotype 2a) that had been isolated from a patient with fulminant hepatic failure were inserted downstream of T7 RNA promoter sequence in pUC19 plasmids.

In particular, the RT-PCR fragments obtained by amplifying viral RNA of JFH-1 strain were cloned into pGEM-T EASY vectors (Promega) to obtain plasmids, pGEM1-258, pGEM44-486, pGEM317-849, pGEM617-1323, pGEM1141-2367, pGEM2285-3509, pGEM3471-4665, pGEM4547-5970, pGEM5883-7003, pGEM6950-8035, pGEM7984-8892, pGEM8680-9283, pGEM9231-9634 and pGEM9594-9678 (see Non-patent document 6). The viral genomic RNA-derived cDNAs contained in such plasmids were ligated together by using PCR method and restriction enzymes to clone the full length viral genomic cDNA. The T7R RNA promoter sequence was inserted upstream of the full length viral genomic cDNA. Hereinafter, the plasmid DNA constructed in this way is referred to as pJFH1 (upper part of Fig 1). The preparation of JFH-1 clone described above has been described in Patent Document 1 and Non-Patent Document 3. Further, the nucleotide sequence of the full length cDNA of JFH-1 clone is registered in international DNA data bank (DDBJ/EMBL/GenBank) with Accession No. AB047639.

Next, plasmid DNA pFGREP-JFH1 was constructed by inserting the EMCV-IRES

(internal ribosome entry site of encephalomyocarditis virus) and the neomycin resistant gene (neo; also referred to as neomycin phosphotransferase gene) between the 5' untranslated region and the core region of pJFH1 plasmid DNA (lower part of Figure 1). This construction procedure was according to the previous publication (Non-Patent Document 4). Further, mutant plasmid clones pJFH1/GND and pFGREP-JFH1/GND were prepared by introducing a mutation which changed the amino acid motif GDD, which corresponded to the active center of RNA polymerase encoded by the NS5B region in pJFH1 and pFGREP-JFH1, to GND. Since the amino acid sequence of the active site of the NS5B protein coded by the mutant clones pJFH1/GND and pFGREP-JFH1/GND is changed, active NS5B protein which is needed for replicating the replicon RNA can not be expressed from the mutant clones.

Further, pFGREP-JFH1/Luc was prepared as a reporter gene-introduced expression vector by inserting the luciferase gene between the MluI site of 415th to 420th and the PmeI site of 2075th to 2082nd of pFGREP-JFH1 to replace the neomycin resistant gene of pFGREP-JFH1 with the luciferase gene. Also, a mutant pFGREP-JFH1/Luc/GND, in which the GDD motif of the active center of NS5b RNA polymerase was changed to GND, was prepared by mutating G at 10933rd of pFGREP-JFH1/Luc to A.

pFGREP-JFH1/EGFP, in which the neomycin resistant gene of pFGREP-JFH1 was replaced with the green fluorescent protein gene, was prepared by inserting the green fluorescent protein gene between the MluI site of 415th to 420th and the PmeI site of 1142nd to 1149th of pFGREP-JFH1. Also, a mutant pFGREP-JFH1/EGFP/GND, in which the GDD motif of the active center of NS5b RNA polymerase was changed to GND, was prepared by mutating G at 10000th of pFGREP-JFH1/EGFP to A.

pFGREP-JFH1/SEAP was prepared by inserting the secretary placental alkaline phosphatase gene between the MluI site of 415th to 420th and the PmeI site of 1982nd to 1989th of pFGREP-JFH1 to replace the neomycin resistant gene of pFGREP-JFH1 with the secretary placental alkaline phosphatase gene. Also, a mutant pFGREP-JFH1/SEAP/GND, in which the GDD motif of the active center of NS5b RNA polymerase was changed to GND, was prepared by mutating G at 10840th of pFGREP-JFH1/SEAP to A.

(B) Preparation of full length HCV genomic RNA and full length HCV replicon RNA

The expression vectors constructed as above, pJFH1, pJFH1/GND, pFGREP-JFH1 and pFGREP-JFH1/GND were digested with restriction enzyme XbaI to prepare template DNAs for the synthesis of the full length HCV genomic RNA and full length HCV replicon RNA. Subsequently 10-20 μg each of XbaI fragment was treated with 20 U of Mung Bean Nuclease in 50 μl reaction solution by incubating at 30°C for 30 min. Mung Bean Nuclease is an enzyme which catalyzes a reaction that involves selectively digesting single strand parts of double stranded DNA. Normally, if RNA is synthesized using the above XbaI fragments as it is as templates, replicon RNAs having 4 extra-bases of CUGA, which is a part of the XbaI recognition site, at 3' terminus are synthesized. Therefore, in this example, 4 bases of CUGA were removed from the XbaI fragments by treating the XbaI fragments with Mung Bean Nuclease. Subsequently, the post-Mung Bean Nuclease treatment solution containing the XbaI fragments was subjected to standard protein removal treatment to obtain purified XbaI fragments without the 4 bases, CUGA, as the template DNA to be used below.

Next, RNA was synthesized in vitro from this template DNA using T7 RNA polymerase. A MEGAscript (Ambion Co.) was used for the RNA synthesis. 20 μl reaction mixture containing 0.5-1.0 microgram of the template DNA was reacted according to the instruction of the manufacturer.

After the RNA synthesis, DNase (2U) was added to the reaction mixture and reacted at 37°C for 15 minutes, and then RNA was extracted with acid-phenol treatment to remove the template DNA. RNAs synthesized in this way from the above template DNAs derived from pJFH1, pJFH1/GND, pFGREP-JFH1 and pFGREP-JFH1/GND were referred to as rJFH1, rJFH1/GND, rFGREP-JFH1 and rFGREP-JFH1/GND, respectively. The nucleotide sequences of these RNAs are shown in SEQ ID NO: 12, 13, 14 and 15 for rJFH-1, rFGREP-JFH1, rJFH1/GND and rFGREP-JFH1/GND, respectively. rJFH1 is an example of the full length HCV genomic RNAs of the present invention which has the same sequence structure as the full length HCV genome of JFH-1 strain. rFGREP-JFH1 is an example of the full length HCV replicon RNA of the present invention.

Subsequently, rFGR-JFH1/Luc (SEQ ID NO:21), rFGR-JFH1/Luc/GND ((SEQ ID

NO:22), rFGR-JFH1/EGFP (SEQ ID NO:23), rFGR-JFH1/EGFP/GND (SEQ ID NO:24), rFGR-JFH1/SEAP (SEQ ID NO:25) and rFGR-JFH1/SEAP/GND (SEQ ID NO:26), which were HCV replicon RNAs, were produced by using as templates the expression vectors prepared as above, pFGREP-JFH1/Luc, pFGREP-JFH1/Luc/GND, pFGREP-JFH1/EGFP, pFGREP-JFH1/EGFP/GND, pFGREP-JFH1/SEAP and pFGREP-JFH1/SEAP/GND, respectively.

Example 2: Replication of the full length HCV genomic RNA in cell and generation of virus particles

(C) Replication of the full length HCV genomic RNA in cell and generation of virus particles

Various amount of the full length HCV genomic RNA (rJFH1 or rJFH1/GND) synthesized as above was mixed with total RNA extracted from Huh7 cells to bring the amount of RNA up to 10 μg. Subsequently the mixed RNA was introduced into Huh7 cells by electroporation method. Huh7 cells subjected to the electroporation treatment were seeded in culture dishes. After incubating for 12, 24, 48 and 72 hours, cells were collected, RNA was extracted and analyzed by the Northern blot method. The Northern blot analysis was carried out according to Molecular Cloning, A laboratory Manual, 2nd edition, J. Sambrook, E.F. Fritsch, T. Maniatis, Cold Spring Harbor Laboratory Press (1989). In particular, RNA extracted from cells after the incubation was subjected to denaturing agarose gel electrophoresis and RNA was transferred to a positively charged nylon membrane after the electrophoresis. ^{32}P labeled DNA or RNA probe prepared from pJFH1 was hybridized to the aforementioned RNA transferred on the membrane. The membrane was washed and exposed to a film to detect RNA bands specific to the full length HCV genomic RNA of JFH-1 clone.

As shown in Figure 2, when rJFH1/GND was transfected into the cells, band of the introduced RNA was confirmed as a weak signal at 4 hours after the transfection, but the signal was getting weaker with the passage of time and the signal from the band was almost undetectable at 24 hours after the transfection. In contrast, when rJFH1 was transfected, the signal intensity of band of the introduced RNA was weakened at first as was the case of

rJFH1/GND between 4-12 hours after the transfection but clear signal of the RNA band was confirmed after 24 hours of the transfection. The confirmed signal was specific to the HCV genomic RNA. That is, it was considered that some introduced full length HCV genomic RNAs were replicated and grown. No replication was observed for rJFH1/GND, in which the active motif of NS5B that is RNA replicative enzyme was mutated, indicating that the activity of NS5B is important for the replication of the full length HCV genomic RNA. Further, same experiments were carried out for the full length genomic RNA derived from hepatitis C virus such as H77 strain (Non Patent Document 7), J6 strain (Non Patent Document 8) and JCH1 strain which was isolated from chronic hepatitis by the present inventors (Non Patent Document 6), all of which had been isolated earlier, but no replication of the full length HCV genomic RNA was confirmed for these strains.

(D) Detection of HCV virus particles in culture medium of transfected cell culture

The electroporation-treated Huh7 cells as described above were seeded in culture dishes and cultured for 12, 24, 48 and 72 hours and then HCV core protein was assayed in the culture supernatant. The assay was carried out according to the Ortho HCV antigen IRMA test (Non Patent Document 9). As shown in Figure 3, the core protein was detected in the culture supernatant 48 and 72 hours after the transfection with rJFH1. To examine whether this core protein is secreted as virus particles, the culture medium 72 hours after the transfection with rJFH1 was fractionated through the sucrose density gradient. In a centrifuge tube 2 ml of 60% (wt/wt) sucrose solution (dissolved in 50 mM Tris pH7.5/0.1M NaCl/1 mM EDTA), 1 ml of 50% sucrose solution, 1 ml of 40% sucrose solution, 1 ml of 30% solution, 1 ml of 20% sucrose solution and 1 ml of 10% sucrose solution were layered and 4 ml of the sample culture supernatant was overlaid thereon. This was centrifuged in a Beckman rotor SW41 Ti at 400,000 RPM, at 4°C for 16 hours. After the centrifugation, this was collected in fractions of 0.5 ml each from the bottom of the tube. The density, the concentration of HCV core protein and the amount of full length HCV genomic RNA in each fraction were determined. Detection of the full length HCV genomic RNA with a quantitative RT-PCR method was carried out by detecting RNA of the 5' untranslated region

of the full length HCV genomic RNA, according to Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, Kawaguchi R, Tanaka S, Kohara M, "Real-Time detection system for quantification of Hepatitis C virus genome", Gastroenterology 116: 636-642 (1999). In particular, the full length HCV genomic RNA contained in RNA extracted from the cell was PCR amplified using synthetic primers, R6-130-S17: 5'-CGGGAGAGCCATAGTGG-3' (SEQ ID NO:16), R6-290-R19: 5'-AGTACCACAAGGCCTTTCG-3' (SEQ ID NO:17) and TaqMan Probe: R6-148-S21FT, 5'-CTGCGGAACCGGTGAGTACAC-3' (SEQ ID NO:18), and EZ rTth RNA PCR kit, and then detected by ABI Prism 7700 sequence detector system.

As shown in Figure 4, the peak of core protein coincided with that of the full length HCV genomic RNA in the fraction 11. The density of this fraction was about 1.18 mg/ml and it indicated a lower specific gravity than that of the conjugate of core protein and nucleic acid reported so far. Further, when similar fractionation was carried out after treating the culture supernatant with 0.25% NP40, the peaks of core protein and the full length HCV genomic RNA were shifted to a specific gravity of about 1.28 mg/ml. That is, it was considered that the NP40 treatment stripped off the surface membrane, which contained lipid and then had a lower specific gravity, from virus particles yielding core particles comprised of only nucleic acid and core protein, and therefore the specific gravity was increased. Above results showed that the full length HCV genomic RNA was replicated in the cell by transfecting rJFH1 into Huh7 cells and, as a result, the virus particles were formed and secreted into the culture supernatant.

Example 3

(E) Preparation of the full length HCV replicon RNA-replicating cell and establishment of the cell clones

The full length HCV replicon RNA-replicating cells were prepared by transfecting rFGREP-JFH1 and rFGREP-JFH1/GND, which were prepared in Example 1, into Huh7 cells as described in Example 2, and then an attempt was made to establish full length HCV replicon RNA-replicating cell clones.

First, after transfecting rFGREP-JFH1 and rFGREP-JFH1/GND respectively into

Huh7 cells, the cells were seeded in culture dishes. After culturing 16-24 hours, G418 was added at various concentrations. Culturing was continued while changing the medium twice a week. After culturing for 21 days, surviving cells were stained with crystal violet. The stained colonies were counted, and the number of resulting colonies per weight of RNA used for transfection was calculated. The culturing was also continued for some of the culture dishes to clone colonies of the surviving cells. RNA, genomic DNA and proteins respectively were extracted from the cloned cells, and then detection of the full length HCV replicon RNA, integration of the neomycin resistant gene into the genomic DNA and the expression of HCV protein were investigated. These results are shown below in detail.

(F) Colony formation ability

The results of above transfection indicated that the colony formation ability per 1 μg of replicon RNA used for transfection was 368 CFU (Colony Forming Unit) /μg RNA, for Huh7 cells transfected with rFGREP-JFH1, at a G418 concentration of 1.0 mg/ml (the left part of Figure 5). In contrast, no colony formation was observed for Huh7 cells transfected with rFGREP-JFH1/GND (the right part of Figure 5). This indicates that the colony formation ability of Huh7 cells transfected with rFGREP-JFH1 replicon RNA relies on the activity of NS5B (RNA polymerase) that is expressed from rFGREP-JFH1. That is, it was considered that in the colony forming cells, the growth of cell became possible as the result of maintenance of G418 resistance due to the continuous expression of the neomycin resistant gene caused by the autonomous replication of rFGREP-JFH1 replicon RNA by means of the action of NS5B expressed from rFGREP-JFH1.

(G) Detection of the full length HCV replicon RNA in established cell clones

Total RNA was extracted by the acid-phenol extraction method from full length HCV replicon RNA-replicating cell clones, which has been established by transfecting rFGREP-JFH1 into Huh7 cells according to the above section (E). Subsequently this total RNA was assayed by the Northern blot method. In the method, pFGREP-JFH1 specific probe was used. As controls, total RNA extracted from untransfected Huh7 cells in a similar

manner (in Figure 6, shown as "Huh7"), a sample containing 10^7 copies of replicon RNA synthesized in vitro in addition to the total RNA extracted from Huh7 cells (in Figure 6, shown as "10^7"), and a sample containing 10^8 copies of replicon RNA synthesized in vitro in addition to the total RNA extracted from Huh7 cells (in Figure 6, shown as "10^8") were used. In Figure 6, 1-4 indicate cell clone numbers.

As a result, RNA having the similar size to rFGREP-JFH1 was detected with an rFGREP-JFH1 specific probe (Figure 6). From this result, it was confirmed that the transfected rFGREP-JFH1 replicon RNA was replicated and grown in the cell clone. It was also demonstrated that there was a difference in the amount of replicon RNA among the cell clones. As shown in Figure 6, for example, the amount of replicon RNA in clone 2 was lower than in other clones.

(H) Confirmation of the presence or absence of the integration of the neomycin resistance gene into genomic DNA

For the cell clones 1-8 obtained according to the (E) (shown as FGR-JFH1/2-1 to FGR-JFH1/2-8 in Figure 7), PCR amplification was performed using neomycin resistance gene-specific primers (sense primer, NEO-S3: 5'-AACAAGATGGATTGCACGCA-3' (SEQ ID NO: 19), antisense primer, NEO-R: 5'-CGTCAAGAAGGCGATAGAAG-3' (SEQ ID NO: 20)) and the host cellular genomic DNA extracted from each of the cell clones as a template, in order to confirm that the resistance of each of the cell clones against G418 was not due to the integration of the neomycin resistance gene into the host cellular genome. As a result, as shown in Figure 7, no positive clone showing the amplification of the neomycin resistance gene was observed.

The result of (H) confirmed that the full length HCV replicon RNA was replicated in the cell clones established by transfection of the full length HCV replicon RNA of the present invention.

(I) Detection of HCV protein

Proteins were extracted by a standard procedure from the cell clones established by

transfection of rFGREP-JFH1, and then analyzed by SDS-PAGE and the Western blot method. The cell clones examined in this case were the same as those used in the above section (G). The cell extract obtained through the transient transfection of the prepared full length HCV genomic RNA into Huh7 cells was used as a positive control (shown as JFH-1 in Figures 8, 9 and 10). The cell extract from the clone obtained by transfecting the HCV subgenomic RNA replicon (SGR-JFH1) was used as a negative control for core protein and a positive control for NS3 and NS5a proteins (shown as SGR-JFH1 in Figures 8, 9 and 10). The cell extract from untransfected Huh7 cells was used as a negative control for all proteins (shown as Huh7 in Figures 8, 9 and 10). Protein samples extracted from each cell clone were blotted onto PVDF membranes (Immobilon-P, Millipore), and then core protein and NS3 protein encoded by the full length HCV replicon RNA therein were detected using an anti-core specific antibody and an anti-NS3 specific antibody (gifted by Dr. Moradpour; Wolk B, et al, J. Virology, 2000, 74: 2293-2304). As shown in Figures 8 and 9, for the cell clones 1-4, which were established by transfecting rFGREP-JFH1, protein of the same size as that of the positive control was detected for each protein. Since neither core protein nor NS3 protein was detected for the untransfected Huh7 cells, it was confirmed in the cell clones 1-4 that the full length HCV replicon RNA, which has been transfected, replicated autonomously and that core protein and NS3 protein were expressed.

Further, for each cell clone, for which the expression of NS3 protein has been confirmed as described above, the expression of NS5A protein from the full length HCV replicon RNA was also confirmed using a serum from a hepatitis C patient as an antibody (Figure 10).

From the results of (H) and (I) described above it was confirmed that in the cell clones, which have been established by transfecting the full length HCV replicon RNA, the full length HCV replicon RNA was replicated and that the viral proteins were also expressed.

(J) Virus particle production in the full length HCV replicon RNA-replicating cells

rFGREP-JFH1 was transfected into Huh7 cells according to the above section (E), the full length HCV replicon RNA-replicating cell clones 2 and 3 (FGR-JFH1/2-3) were

established, and then their culture supernatants were recovered. HCV virus particles were assayed in the culture supernatants according to a similar method to (D) described above. The result is shown in Figure 11. In Figure 11, a shaded circle represents specific gravity (g/ml) of each fraction. A closed circle represents an amount of core protein (fmol/L). A open circle represents a titer of the full length HCV replicon RNA (x 0.1 copy/mL).

As shown in Figure 11, the peak of core protein coincided with that of the full length HCV replicon RNA in the fractions having specific gravities of about 1.18-1.20 mg/ml. A small peak was also found in the lighter fraction. From the above results it is shown that the full length HCV replicon RNA was replicated in Huh7 cells transfected with rFGREP-JFH11, and virus particles were formed and secreted into the culture supernatant thereof.

Example 4

(K) Infection experiment with virus particles in culture supernatant

Huh7 cells were infected with virus particles in culture supernatant by adding each culture supernatant of cell clones 1-8 used in (H) (i.e., FGR-JFH1/2-1, FGR-JFH1/2-2, FGR-JFH1/2-3, FGR-JFH1/2-4, FGR-JFH1/2-5, FGR-JFH1/2-6, FGR-JFH1/2-7, FGR-JFH1/2-8) to Huh7 cells. On the next day G418 was added at 0.3 mg/ml to the culture media of the infected Huh7 cells, and the Huh7 cells were further cultured for 21 days. After the end of culturing, cells were fixed and stained with crystal violet. Colony formation was observed for cells infected with the culture supernatants of FGR-JFH1/2-3, FGR-JFH1/2-5 and FGR-JFH1/2-6, respectively. On the other hand, no colony formation was observed for cells infected with the culture supernatant of SGR-JFH1/4-1, subgenomic replicon cells (described in Non Patent Document 6), used as a control. Figure 12 shows a photograph of a stained culture dish after culturing for 21 days with the added 4 ml or 8 ml of the culture supernatant of FGR-JFH1/2-3 or SGR-JFH1/4-1. Three and nine colonies were found in the dish in which the cells mixed with 4 ml and 8 ml of the culture supernatant of FGR-JFH1/2-3 had been seeded, respectively. However, no colony was observed in the dish, in which the cells mixed with the culture supernatant of SGR-JFH1/4-1 had been seeded.

Subsequently, colonies formed by infecting with hepatitis C virus using the culture

supernatant of FGR-JFH1/2-3 and FGR-JFH1/2-5, respectively, were cloned. Three clones of FGR-JFH1/C2-3-11, FGR-JFH1/C2-3-12 and FGR-JFH1/C2-3-13 were established from the culture dish infected with the culture supernatant of FGR-JFH1/2-3, and 2 clones of FGR-JFH1/C2-5-11 and FGR-JFH1/C2-5-12, were established from the culture dish infected with the culture supernatant of FGR-JFH1/C2-5.

When Huh7 cells were infected with the culture supernatant of each cell clone of FGR-JFH1/C2-3-11, FGR-JFH1/C2-3-12, FGR-JFH1/C2-3-13, FGR-JFH1/C2-5-11 and FGR-JFH1/C2-5-12, colony formation was observed in culture dishes infected with the culture supernatant of FGR-JFH1/C2-3-12 and FGR-JFH1/C2-5-12, respectively. From the cells infected with the culture supernatant of FGR-JFH1/C2-3-12, additional 2 clones of FGR-JFH1/C2-3-12-1 and FGR-JFH1/C2-3-12-2 were established. From the cells infected with the culture supernatant of FGR-JFH1/C2-5-12, additional 2 clones of FGR-JFH1/C2-5-12-1 and FGR-JFH1/C2-5-12-2 were established.

RNA, protein and genomic DNA were extracted from these cell clones which had been established from cells infected with the culture supernatant of the full length HCV replicon RNA-replicating cells. Examination for the integration of the neomycin resistant gene into the genomic DNA of these cell clones by PCR using the genomic DNA as a template resulted in all negative. Furthermore, the full length HCV replicon RNA that is replicating in the cells could be detected by the quantitative PCR using RNA as a template. Still further, core protein could be detected in the culture supernatant. These results indicate that the virus particles containing the full length HCV replicon RNA which are produced by the full length HCV replicon RNA-replicating cell of the present invention can infect another cell.

Industrial Applicability

According to the method of the present invention, HCV virus particles can be prepared in a cell culture system. By using the replicon RNA of the present invention, RNA containing the full length HCV genomic RNA can be produced efficiently in a cell culture system. Furthermore, by using the cells, in which the full length HCV replicon RNA or the full length HCV genomic RNA according to the present invention is introduced, the full length

HCV replicon RNA or the full length HCV genomic RNA can be replicated, and the HCV virus particles of the present invention can be produced continuously in the cell culture system. The cells, in which the full length HCV replicon RNA or the full length HCV genomic RNA according to the present invention is introduced, can also be used as a test system for screening various substances which influence the process of HCV replication, virus particle formation and extracellular release of virus particles. The full length HCV replicon RNA and full length HCV genomic RNA, and virus particles of the present invention are also useful as a viral vector for a foreign gene. The virus particles of the present invention or a part thereof can be included into a vaccine as the vaccine antigen against hepatitis C virus. Further, the system, in which the virus particles of the present invention and other cells are cultured together, can be utilized as a test system for screening various substances which have an influence on the infection of cells with virus particles. The full length HCV replicon RNA or the full length HCV genomic RNA of the present invention is useful as a template which enables simple reproduction of the HCV full length genome sequence.

All publications, patents and patent applications cited herein are incorporated herein by reference in their entirety.

SEQUENCE LISTING FREE TEXT

SEQ ID NO: 1 represents the sequence of the 5' untranslated region of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 2 represents the core protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 3 represents the E1 protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 4 represents the E2 protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 5 represents the NS2 protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 6 represents the NS3 protein-coding sequence of HCV genomic RNA derived

from JFH-1 clone.

SEQ ID NO: 7 represents the NS4A protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 8 represents the NS4B protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 9 represents the NS5A protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 10 represents the NS5B protein-coding sequence of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 11 represents the sequence of the 3' untranslated region of HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 12 represents the sequence of the full length HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 13 represents the sequence of the replicon RNA comprising the full length HCV genomic RNA derived from JFH-1 clone.

SEQ ID NO: 14 represents the sequence of the full length HCV genomic RNA derived from JFH-1 clone in which the amino acids motif GDD has been mutated into GND.

SEQ ID NO: 15 represents the sequence of the replicon RNA comprising the full length HCV genomic RNA derived from JFH-1 clone in which the amino acids motif GDD has been mutated into GND.

SEQ ID NOs: 16-20 represent the sequences of primers.

SEQ ID NO: 21 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/Luc.

SEQ ID NO: 22 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/Luc/GND.

SEQ ID NO: 23 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/EGFP.

SEQ ID NO: 24 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/EGFP/GND.

SEQ ID NO: 25 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/SEAP.

SEQ ID NO: 26 represents the sequence of the replicon RNA derived from an expression vector pFGREP-JFH1/SEAP/GND.